Mª Paz Lorenzo[1]
Alma Villaseñor[1]
Anuradha Ramamoorthy[2]
Antonia Garcia[1]

[1]Center for Metabolomics and Bioanalysis (CEMBIO), Facultad de Farmacia, Universidad CEU San Pablo, Campus Montepríncipe, Madrid, Spain
[2]Laboratory of Clinical Investigation, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, MD, USA

Research Article

Optimization and validation of a capillary electrophoresis laser-induced fluorescence method for amino acids determination in human plasma: Application to bipolar disorder study

Quantitative and qualitative analysis of amino acids in biofluids offers relevant information in diagnosis of diseases, evaluation of nutritional state, and in elucidating metabolic influences on physiology. A simple, rapid, and robust procedure in terms of sample treatment, separation, and quantitation based on CE-LIF has been optimized for use in human plasma samples. Time required for derivatization was 15 min and analysis time was 35 min. 4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was the labeling agent used for obtaining fluorescent derivatives. Electrophoretic conditions were: 175 mM borate buffer at pH 10.25 prepared with 12.5 mM β-cyclodextrin. The voltage applied was +21 kV. Fourteen amino acids could be quantified: L-proline, L-phenylalanine, L-leucine, L-isoleucine, L-ornithine, D-ornithine, L-glutamine, L-alanine, L-threonine, glycine, L-serine, D-serine, taurine and L-glutamate. With this chiral CE-LIF method, L- and D-amino acids are adequately separated. The method was validated for a representative group of amino acids in human plasma: L-proline, L-isoleucine, L-ornithine, L-glutamine, L-alanine L-threonine, glycine, L-serine, D-serine, and glutamate. The method has been successfully applied to human plasma from patients with bipolar disorder, all of whom were taking lithium as a mood stabilizer. Eleven amino acids were quantified in plasma from nine patients, aged 24–55 years. The results were in accordance to published values for the bipolar patients. The method is useful particularly in studies where plasma amino acid levels can be used as biomarkers for diagnosis of diseases, evaluating the disease progression, and monitoring response to drug therapy.

Keywords:
Amino acids / Biofluid / Bipolar disorder / Fluorescence / Plasma
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1 Introduction

Amino acids are organic compounds that play a major role in a number of important biological processes including energy metabolism, neurotransmission, lipid transport, etc. Quantitative and qualitative analysis of amino acids has been applied in the diagnosis of diseases (e.g. inborn errors of metabolism), evaluation of nutritional state, and in elucidating metabolic influences on physiology [1–3]. Plasma levels of amino acids have been shown to be associated with symptoms and severity of a number of neuropsychiatric diseases such as schizophrenia, depression, and bipolar disorder (BD) [3, 4]. BD, formerly known as manic-depressive psychosis, is one of the most debilitating and common psychiatric disorders worldwide. BD is well distinguished by flashing emotional and behavioral disruptions [5]. As free amino acids are important for neurotransmission and receptor function, changes in their metabolism can be used not only for disease diagnosis, but also for monitoring treatment outcomes. Over or under expression of specific amino acids have been observed in patients of neuropsychiatric conditions when compared to controls: for example, some authors have found higher concentrations of glutamate, glutamine, and glycine in BD patients [4,6]; depressed patients had a significant increase in plasma levels of glutamate, glycine, glutamine, and taurine when compared to controls [3]. Studies have suggested that

Correspondence: Dr. Antonia García, Center for Metabolomics and Bioanalysis (CEMBIO), Facultad de Farmacia, Universidad CEU San Pablo, Campus Montepríncipe, 28668 Boadilla del Monte (Madrid), Spain
E-mail: antogar@ceu.es
Fax: +34-91-3724712

Abbreviations: BD, bipolar disorder; **FQ**, 3-(2-furoyl) quinoline-2-carboxaldehyde; **IS**, internal standard (aminoadipic acid); **NBD-F**, 4-fluoro-7-nitro-2,1,3-benzoxadiazole; **NDA**, naphthalene dicarboxaldehyde; **OPA**, O-phthaldialdehyde

higher plasma serine concentration is a possible biomarker for schizophrenia, mania, paranoia, psychotic depression, and unipolar depression [7–9]. Thus, these neuroactive amino acids can be utilized as biomarkers in the diagnosis and prognosis of a number of neurological disorders. Some of these amino acids are present in trace levels in biological fluids. Increased complexity of the biological matrices makes it even more difficult to quantify these amino acids [10]. Thus, it is necessary to develop a highly sensitive analytical methodology for the determination of these amino acids. As was described elsewhere [11], quantitative measurement of the complete range of amino acids in biological samples is an important challenge in clinical biochemistry for several reasons: (i) amino acids do not have a chromophore; (ii) most of them are highly hydrophilic and therefore are difficult to extract using organic solvents for GC, show poor retention in RP-LC, and are difficult to separate from the solvent peak. The chromatographic separation using RP needs analyte derivatization or the use of ion-paring agents to increase the chromatographic retention of analytes and to preclude their co-elution with the void volume; (iii) in GC, the derivatized compounds are very volatile, usually resulting in more than one derivative per analyte and some of these are lost during the sample treatment. Although, GC-MS involves time-consuming derivatization or complicated extraction procedures, it is the gold standard technique for the diagnosis of inborn errors of metabolism.

Different analytical techniques based on chromatographic and electrophoretic methodologies have been reported in the literature for analyzing amino acids from different biological matrices. Among the LC techniques, both ion exchange, RP-HPLC, and RP-HPLC-MS are used extensively. Other common LC based methods include hydrophilic interaction chromatography [12]. Different detectors can been used with LC including UV [13, 14], fluorescence [15, 16], and MS [17]. With UV-VIS and fluorescence detection, derivatization (pre or post separation) is often necessary due to the lack of a chromophore [18, 19]. Ion exchange separation usually requires a postcolumn derivatization with ninhydrin [18]. *O*-phthaldialdehyde (OPA) in combination with 2-mercaptoethanol is used to produce fluorescent isoindole derivatives that can be separated by HPLC and detected fluorimetrically. However, this reagent reacts only with primary amines in the presence of thiol and generates unstable derivatives [20]. For chiral analysis, by substituting a chiral thiol reagent for 2-mercaptoethanol, diasteromeric derivatives are produced and can be separated by HPLC [16].

Another technique that is routinely used to detect changes in the metabolic profiles is CE. Some reviews described advances in amino acid analysis by CE [21–23]; according to some of them, LIF provides the lowest reported LODs among the detection methods available for use with CE [24]. Several labeling techniques can be used with CE-LIF; each of these has its own strength and drawbacks. As reviewed previously [25], six different labeling agents are commonly used: NDA (naphthalene dicarboxaldehyde) [26], OPA [27], FQ (3-(2-furoyl)quinoline-2-carboxaldehyde) [28–30] and fluorescein derivatives such as FITC (fluorescein-

5-isothiocyanate) [31, 32] or carboxyfluorescein succinimidyl ester [33]. The main drawbacks are the use of toxic chemicals such as sodium cyanide (NDA, FQ, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA)), the expensive UV laser (OPA, NDA) [34], and the generation of a large number of fluorescein related labeling by-products. Moreover, FQ derivatives are not soluble enough in aqueous buffers [35]. Also, NDA, OPA, CBQCA, and FQ only react with primary amines; proline, a secondary amine cannot be labeled with these reagents.

4-Fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) is a labeling agent that can be used for both primary and secondary amines. Sensitive detection of NBD-derivatives can be performed using HPLC and CE coupled to common argon ion laser [36]. When compared to fluorescein-based dyes, the advantages of using NBD-F are the short time required to achieve derivatization and the cleaner electropherograms due to low number of by-products. Tseng et al. [37] described a MEKC method based on NBD-F with a BGE containing sodium cholate, β-cyclodextrin (CD), Brij 35 in aqueous borate buffer, pH 9.3 containing 7% methanol; they applied this method to quantify amino acids in biofluids, and this includes a complex extraction procedure and complicated electropherograms. We recently validated a bioanalytical method based on CE-LIF using NBD-F as a derivatizing agent for the analysis of amino acids in urine and hippocampus tissue [11]. To our knowledge, there are no other published methods for the analysis of amino acids in plasma or serum by CE-LIF using NBD-F. We have also quantified the L-and D-serine enantiomers using the current methodology using the labeling agent, NBD-F. A previous study by Singh et al. [32] has used FITC to quantify selected amino acid enantiomers including serine. However, none of the previously reported CE based methods have been applied to study amino acid concentrations in plasma samples from psychiatric patients.

The goal of this work was to optimize and validate a method for analyzing amino acids in plasma by a CE method combining chiral selector and LIF detection. This validated method was applied to measure selected amino acids (L-proline, L-phenylalanine, L-leucine, L-isoleucine, L-ornithine, L-glutamine, L-alanine, L-threonine, glycine, L-serine, D-serine, taurine and glutamate) using plasma samples of patients with BD.

2   Materials and methods

2.1   Chemicals and reagents

Analytical grade standards of L- and D-amino acids: phenylalanine 99%, glutamine 98%, serine 99%, aspartic acid 98%, glutamic acid 99%, valine, alanine, threonine, proline 99%, isoleucine 98%, leucine, ornithine hydrochloride 99%, 2-aminoadipic acid (internal standard--IS), glycine 99%, taurine 99%, β-CD 97%, and boric acid 99.5% were purchased from Sigma–Aldrich (Steinheim, Germany). Hydrochloric acid was from Fluka (Buchs, Switzerland) and sodium

hydroxide from Panreac Química (Barcelona, Spain). NBD-F 99% was from TCI (Tokyo, Japan). All solutions and dilutions were prepared with purified water from a Milli-Qplus 185 system (Millipore, Billerica, MA, USA). Individual 25 mM stock solution of each amino acid was prepared in purified water and stored at $-20°C$. From this, a dilution of 1 mM of each amino acid was prepared and stored at $+4°C$ during the working week. These solutions were diluted as required on the day of the analysis. The derivatization solution of NBD-F was prepared by dissolving 80 mM NBD-F in methanol and mixing with equal parts of 500 μM HCl to give a final concentration of 40 mM NBD-F/250 μM HCl in 50% methanol.

2.2 Instrumentation

CE experiments were carried out on a P/ACE MDQ system (Beckman-Coulter, Fullerton, CA, USA) with LIF detector, an argon source operating at λexc: 488 nm and λem: 522 nm, a capillary silica column (Beckman Coulter, Madrid, Spain) 60 cm in total length, and 75 μm of id. All solutions were kept refrigerated at $7 \pm 2°C$ in the CE autosampler. Data acquisition and instrument control were carried out using 32 Karat™ system software version 7.0 (Beckman Coulter, USA).

At the beginning of its use, the capillary was conditioned by flushing with 1 M NaOH (15 min) and water (15 min). Between the runs, the capillary was flushed with HCl 0.1 M (3 min), water (5 min), and BGE (5 min). Injections were made at the anodic end with a pressure of 0.5 psi (33 mbar) for 10 s. After optimization, running buffer (BGE) consisted of 175 mM borate buffer at pH 10.25 (pH adjusted with 2M NaOH) and 12.5mM β-CD. The voltage applied was +21 kV and the current observed under these conditions was 140 μA. The capillary thermostat was set at 17°C. The buffer vials were refreshed after every sixth analysis to maintain consistency.

2.3 Plasma samples

2.3.1 Study population

The study was approved by the Combined Neuroscience Institutional Review Board of the National Institutes of Health. All subjects provided written informed consent. All subjects were studied as inpatients at the National Institute of Mental Health Clinical Research Center, Mood Disorders Research Unit in Bethesda, Maryland. As described previously [38], patients corresponded to bipolar I or II disorder and were experiencing a major depressive episode without psychotic features were enrolled in the study. This study included nine subjects; one male and eight females, with an age range of 24 to 55 years (mean age = 39.2 (SD 10.5) years). These patients were also taking the mood stabilizer, lithium (0.6–1.2 mEq/L) for 4 wks before and during the study, no other psychotropic medications were taken. Pool of plasma for method development and validation was from healthy volunteers. All samples were stored at $-80°C$ until the day of the assay.

2.3.2 Ultrafiltration

Plasma proteins were removed by filtration using the Centrifree® ultrafiltration devices from Millipore (Ireland; 30 kDa cutoff). A volume of 150 μL of plasma was placed into a single Centrifree® device and centrifuged at $2000 \times g$, 4°C for 60 min. The filtered plasma was then used for amino acid analysis.

2.3.3 Derivatization procedure

In a glass tube, 20 μL of filtered plasma, or the working standard solution was mixed with 20 μL of 200 mM aminoadipic acid (IS), 25 μL of 40 mM NBD-F (derivatization solution), and 150 μL of borate buffer 10 mM, pH 10. The resultant mixture was vortexed for 10 s and metabolite derivatization was performed at 60°C for 15 min in an oven. Immediately after derivatization, samples were kept refrigerated in the CE autosampler at 7°C for at least 30 min before CE-LIF analysis

2.3.4 Identification of amino acids in the plasma

Selected amino acids (L-proline, L-phenylalanine, L-leucine, L-isoleucine, L-ornithine, L-glutamine, L-alanine, L-threonine, glycine, L-serine, D-serine, taurine and glutamate) were analyzed in this study. In order to identify and quantify these amino acids in plasma, the standard solution of the pure amino acid was injected alone, followed by the sample, and then the sample that was spiked with the standard. Moreover, L- and D- pure enantiomers for each amino acid were injected to check whether the final analytical method was capable of separating these isomers.

2.4 Validation study

The method was validated for selected amino acids for selectivity, linearity, accuracy, instrumental precision, method precision (both with standards and samples), LOQ, and LOD. Selectivity was checked by (i) analyzing the profile for ultrafiltered and nonderivatized plasma pool without the labeling agent; no peak was observed in this case; (ii) analyzing a blank containing the derivatizing reagent to distinguish the reagent peaks; and (iii) comparing the electropherograms of derivatized samples with and without the IS to check that there were no others peaks at IS migration time. Linearity was estimated by assaying at least five levels of concentrations of the standards in triplicate, covering all the expected values ranging from 25 to 200% or 300% of mean values found in a preliminary assay. The individual ranges are described in Table 1. Recovery was estimated by comparing in triplicate, the values of spiked samples prepared in a linear range (taking into account the endogenous concentrations that had been previously measured in the samples). Instrumental precision was evaluated by multiple injections ($n = 10$) of a homogeneous derivatized standard solution. Within-day precision of

Table 1. Validation data for selected amino acids in human plasma with the optimized CE-LIF method

	L-Proline	L-Isoleucine	L-Ornithine	L-Glutamine	L-Alanine	L-Threonine	Glycine	L-Serine	D-Serine	L-Glutamate
Linearity ($N = 20$)										
Slope	0.0078 ± 0.0003	0.0059 ± 0.0004	0.0017 ± 0.0002	0.0048 ± 0.0003	$0.00382 \pm 8.4 \times 10^{-5}$	0.0052 ± 0.0003	0.0059 ± 0.0004	0.0055 ± 0.0003	0.0047 ± 0.0002	0.00481 ± 0.00003
Intercept	-0.075 ± 0.099	-0.053 ± 0.067	-0.020 ± 0.028	-0.37 ± 0.27	-0.028 ± 0.038	-0.19 ± 0.12	-0.012 ± 0.018	-0.21 ± 0.13	0.0004 ± 0.003	0.012 ± 0.012
r	0.997	0.992	0.991	0.997	0.999	0.997	0.995	0.996	0.998	0.9997
Range (µM)	25–300	25–300	25–300	125–1500	25–750	25–600	25–750	25–750	0.5–25	25–300
Accuracy										
%	97.1	101.5	97.1	101.8	94.0	96.0	96.7	103.7	100.9	92.0
RSD(%)	3.7	5.2	6.9	4.5	7.8	7.3	6.0.	6.2	4.5	9.8
Instrumental precision ($N = 12$) RSD(%)	4.3	2.2	4.3	5.2	1.4	5.9	4.3	5.6	4.8	1.6
Method precision standard										
Interassay ($N = 12$) RSD (%)	6.5	4.2	6.8	6.2	3.8	5.5	5.2	6.7	4.4	2.8
Intra-assay ($N = 6$) RSD (%)	3.1	3.5	2.9	3.2	3.6	3.7	3.7	3.3	2.7	3.0
Method precision sample										
Interassay ($N = 12$) RSD (%)	6.0	5.3	6.0	5.7	5.8	5.5	4.4	5.3	6.8	3.4
Intra-assay ($N = 6$) RSD (%)	5.4	5.2	4.0	5.6	2.0	2.9	2.8	5.1	5.0	2.0
LOQ (EURACHEM) (nM)	738	630	1.44 µM	146	889	714	128	174	231	203
LOD (nM)	221	189	433	43.7	267	214	38.3	52.2	69.5	61.0

the method was checked by injecting individual preparations of standards and samples in the midrange of the calibration curve. Intermediate precision was tested in the same way, but on a different day, with freshly prepared buffers and reagents. LOQ for the selected amino acids were estimated using the Eurachem method [39] by injecting six replicates of each standard at least four levels of concentration ranging from 0.02–5.0 μM for D-serine and 0.1–20 μM for the rest of the amino acids. LOQ was established by representing RSD of the six replicates versus concentration and interpolating the concentration corresponding to 10%. LOD was calculated by means of the relation LOD: (3/10) × LOQ and checked experimentally.

3 Results and discussion

3.1 Method optimization

The principal goal of this study was to develop and validate a robust and rapid CE-LIF method for the determination of amino acid profile in human plasma. The method was based on our previous CE-LIF method for the analysis of amino acids in urine and hippocampus tissue samples [11], with some optimizations for this new biological matrix, human plasma. This validated method was used to measure selected amino acids in patients with BD.

In order to analyze amino acid concentrations in human plasma by CE-LIF, several optimizations were performed. Plasma proteins were first removed as amino acid profiling requires initial plasma deproteinization in order to avoid capillary clogging and changes due to absorptions in the capillary wall. These procedures involve the use of organic solvents or strong organic acids. The use of acid is also associated with some disadvantages; the labeling reaction (before derivatization with AQC, FITC, or NBD-F etc.) is performed in an alkaline medium by the addition of borate buffer. The acidified sample requires much more buffer for pH switching, thus increasing the ionic strength of the sample. The advantages to be gained from adopting the strategy of ultrafiltration are mainly not introducing any effect on the physicochemical status of the free amino acid fraction in the plasma and not introducing any substances that could affect derivatization. Because of the reasons cited above and based on the data available in the literature [14, 40], ultrafiltration was selected as the method for sample deproteinization. Plasma diluted (1:1) with pure water was compared with undiluted plasma sample. Better sensitivity without signal saturation was observed with the undiluted sample (data not shown). As an initial screen, previously described CE-LIF method [11] was applied to the filtered plasma after derivatization, but an unknown compound co-eluted with L-serine (which was not observed in our previous method). Lower concentrations of borate buffer were tested (90, 70, and 50 mM); decreasing the buffer concentration, analysis time was lowered but resolution equal to zero was achieved, nevertheless L- and D-serine were resolved. Higher concentrations

of borate buffers were assayed: 110, 125, 150, and 175 mM after 30 s of sample injection (Fig. 1). Different sample injection times ranging from 10, 20, 30, 40, and 50 s were also tested, large differences in sensitivity were found. At more than 30 s of sample injection, peaks such as D-serine had higher signal and lower deviation, but a higher risk of peak co-migration was observed near the derivatizing peak. Thus, the 30 s injection time was applied for D-serine, and 10 s sample injection was applied for all other amino acids analyzed. With the use of 110 mM borate buffer (Fig. 1A), L-serine showed a single peak suggesting that the co-eluting compound migrated at the same time. As can be seen from Fig. 1, L-serine was fully resolved with higher concentrations of buffer (175 mM; Fig. 1D) and the peak shape was good. Moreover, both glutamine and alanine were fully resolved when compared to 125 and 150 mM concentration (Fig. 1B and C). Migration time, number of theoretical plates, and resolution were calculated for these two critical pair of peaks (Table 2); best results for resolution >1.5 were obtained with 175 mM BGE.

Addition of methanol or ACN to the BGE (up to 20% v/v was studied as the fluorescence of NBD derivatives is very sensitive to the hydrophobicity of the BGE, but higher migration time in the whole profile and wider peaks were obtained. The final conditions for the optimized CE-LIF method were: running buffer 175 mM borate buffer at pH 10.25, 12.5 mM β-CD, 10 s of sample injection (33 mbar), L-2-aminoadipic acid (IS using ultrafiltered, undiluted plasma samples.

The last step before validation was the identification of the selected amino acids in the plasma. As described in the method section, migration times and peak areas were compared between the ultrafiltered sample, pure standard and spiked sample (Fig. 2). Fourteen amino acids that were identified in the plasma profile include, L-proline, L-phenylalanine, L-leucine, L-isoleucine, L-ornithine, D-ornithine, L-glutamine, L-alanine, L-threonine, glycine, L-serine, D-serine, taurine, and glutamate. Finally, for the identified amino acids pure L- and D-enantiomers were injected to ensure that the method could separate the enantiomers. The enantiomers of all these amino acids were satisfactorily separated. As a chiral selector was included in the BGE, chiral separation of the standards with resolution ≥1.5 was achieved (data not shown).

3.2 Validation

A complete validation was performed for only a representative group of amino acids including, L-proline, L-isoleucine, L-ornithine, L-glutamine, L-alanine, L-threonine, glycine, L- and D- serine, and L-glutamate. A summary of the validation parameters for the selected amino acids are shown in Table 1.

During validation, standards fit the linear model (r > 0.99) for all amino acids and no bias was found for most of them excluding, L-glutamine, L-threonine, and L-serine. However, in spite of the bias, no practical consequence was seen in the recovery. The recoveries ranged from 92.0 to 103.7%
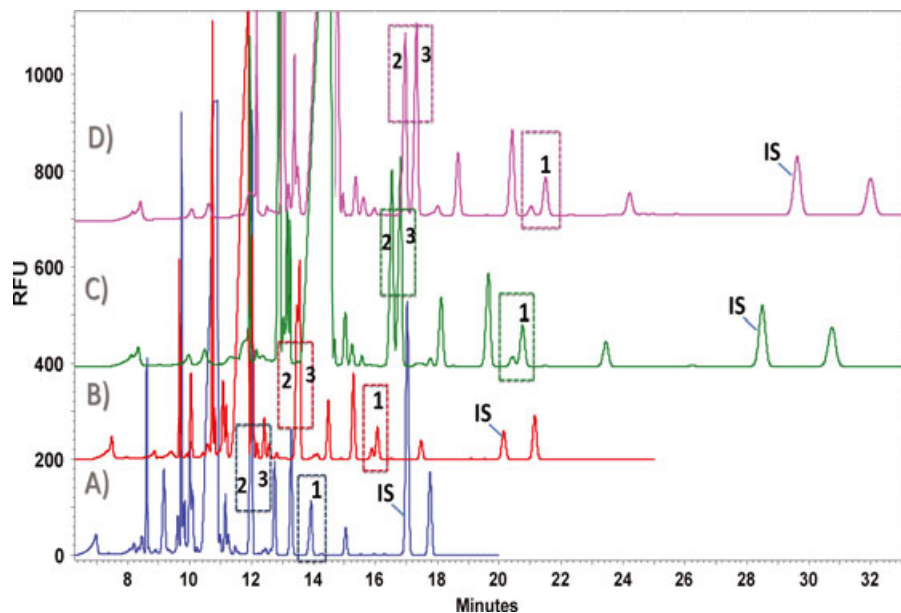
Figure 1. Influence of buffer concentration on amino acid profile; borate buffer, pH 10.25 and 12.5 mM β-CD, 30 s of sample injection. (A) 110 mM, [c.o. = 88 μA]; (B) 125 mM, [c.o. = 100 μA]; (C) 150 mM, [c.o. = 119 μA]; (D) 175 mM, [c.o. = 136 μA]. Key. [c.o.] = Current; [1] = L-serine; [2] = L-glutamine; [3] = L-alanine; [IS] = L-2-aminoadipic acid (A. 500 μM, B. 100 μM, C. and D. 250 μM).

Table 2. Performance parameters on critical separations of glutamine/alanine and unknown/L-serine through the increase of running buffer concentration

Analyte	Buffer 110 mM [c.o. = 88 μA]			Buffer 125 mM [c.o. = 100 μA]			Buffer 150 mM [c.o. = 119 μA]			Buffer 175 mM [c.o. = 136 μA]		
	tm (min)	N(USP)	R(USP)	tm (min)	N(USP)	R(USP)	tm (min)	N(USP)	R(USP)	tm (min)	N(USP)	R(USP)
Gln	12.0	139 113	0.0	13.5	29 080	0.3	15.5	160 923	1.1	17.0	101 646	1.7
Ala	12.0			13.6	104 822		15.7	161 776		17.3	98 086	
Unknown	13.9	110 615	0.0	15.9	148 677	1.1	18.9	140 663	1.1	21.0	94 944	1.7
L-Ser	13.9			16.1	155 873		19.1	151 890		21.5	95 162	

The amino acids with higher concentration range from 220–590 μM were L-glutamine, L-alanine, and glycine. Amino acids such as L-threonine, L-serine, L-ornithine, L-proline, L-isoleucine, taurine, and L-glutamate had a concentration range of 50 to 120 μM while D-serine had a concentration of approximately 2 μM. Abbreviations: Gln: glutamine; Ala: alanine; L-Ser: L-serine; tm: migration time; N: theoretical plates; R: resolution; [c.o.]: current observed.

and the differences were not statistically significant. For standards ($n = 12$), the instrumental precision ranged from 1.4 to 5.9%. Intra-assay precision for standards ranged from 2.9 to 3.7% ($n = 6$) and interassay precision from 2.8 to 6.8% ($n = 12$). Intra-assay precision for samples: six samples prepared from the same pool were treated from the beginning and run in the same assay, the daily RSDs ranged from 2.0 to 5.6% and from 3.4 to 6.8% in different days ($n = 12$). Theoretical LOQ calculated by the Eurachem method for these amino acids ranged from 128 nM for glycine to 1.44 μM for L-ornithine, which were lower than the values observed in the samples.

3.3 Quantitation of amino acids in plasma from BD patients

The validated CE-LIF method was applied to study plasma samples from nine patients diagnosed with BD. Eleven amino acids were determined in all the profiles: L-proline, L-isoleucine, L-ornithine, L-glutamine, L-alanine, L-threonine,

glycine, L- and D-serine, taurine, and L-glutamate. All the results were in μM range and are summarized in Table 3. Interestingly, five patients whose samples were collected from 2007 to 2009 showed lower concentration of L-glutamine and higher concentration of glutamate than the more recent samples. A possible explanation for this is that serum hydrolases may increase the free glutamic acid by breaking down glutamine [41] during storage. Although these samples were stored at −80°C, the length of storage (3–5 years) could have contributed to this effect in both amino acids. Therefore, only the most recent samples were considered for statistical analysis of L-glutamate and L-glutamine. The results obtained and values from the literature are presented in Table 4. Major amino acids with concentrations from 220–590 μM were L-glutamine, L-alanine, and glycine, amino acids at middle concentrations from 50 to 120 μM were L-threonine, L-serine, L-ornithine, L-proline, L-isoleucine, taurine, and L-glutamate, and minor amino acids such as D-serine had a concentration of around 2 μM. Even though previous studies analyzed plasma samples not only from BD but also from other depressed
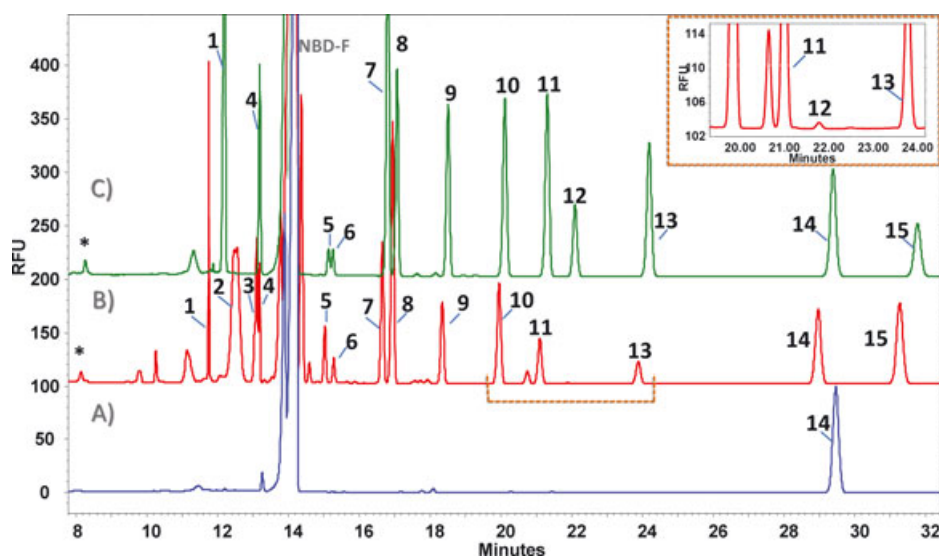
Figure 2. (A) Blank of sample (NBD-F). (B) Plasma profile. (C) Standards. Peak identification: 1. L-proline [200 μM]; 2. L-phenylalanine; 3. L-leucine; 4. L-isoleucine [100 μM]; 5. L-ornithine [100 μM]; 6. D-ornithine [100 μM]; 7. L-glutamine [500 μM]; 8. L-alanine [250 μM]; 9. L-threonine [200 μM]; 10. glycine [200 μM]; 11. L-serine [250 μM]; 12. D-serine [100 μM]; 13. taurine [150 μM]; 14. L-2-aminoadipic acid [IS = 200 μM]; 15. L-glutamate [100 μM]; D. NBD-F hydrolysis products. Conditions: 175 mM borate buffer, pH 10.25 and 12.5 mM β-CD, 10 s of sample injection.

Table 3. Quantitation of amino acids on plasma from BD patients

| Patient | Year | Gender | Age | Plasma concentrations (μM) | | | | |
				L-Proline	L-Isoleucine	L-Ornithine	L-Glutamine	L-Alanine
1	2007	Female	34	70.9	60.0	121.8	476.8	349.2
2	2008	Female	24	36.1	38.7	60.5	403.0	268.1
3	2008	Male	55	67.1	63.3	94.6	560.8	399.9
4	2009	Female	37	73.1	75.4	78.9	471.0	440.1
5	2009	Female	44	63.7	68.0	113.5	354.9	322.5
6	2010	Female	48	41.8	46.2	77.4	633.7[a]	298.3
7	2010	Female	29	23.0	43.3	54.8	531.8[a]	278.8
8	2010	Female	32	56.9	45.4	75.0	433.7[a]	214.4
9	2011	Female	50	57.4	42.7	81.7	698.2[a]	261.1
			Average	54.5	53.7	84.3	574.3	314.7
			SD	16.2	12.4	21.0	116.1	67.4

| Patient | Sample year | Plasma concentrations (μM) | | | | | |
		L-Threonine	Glycine	L-Serine	D-Serine	L-Glutamate	Taurine
1	2007	136.7	209.3	125.2	1.3	130.5	57.0
2	2008	93.4	197.1	93.6	1.7	123.1	33.3
3	2008	134.7	251.7	126.4	2.3	85.5	124.8
4	2009	116.0	192.7	85.1	4.5	211.5	55.3
5	2009	99.9	209.3	92.5	1.9	193.1	79.8
6	2010	177.5	247.5	109.4	3.5	22.4[a]	26.5
7	2010	120.2	380.1	94.3	2.7	27.5[a]	55.6
8	2010	131.7	145.2	73.4	1.2	27.7[a]	21.3
9	2011	135.4	254.5	110.0	2.8	35.5[a]	24.0
	Average	127.3	231.9	101.1	2.4	28.3	53.1
	SD	23.2	61.8	16.9	1.0	5.4	31.3

a) Values of L-glutamine and L-glutamate considered for statistical analysis.

patients undergoing lithium or other treatments, our results are concordant with the literature. The glutamate and glycine results were very similar to the previous values reported for patients suffering from major depressive disorder [42] and BD patients [43]. Taurine values were lower compared with previously studied depressed patients [3, 44], but comparable levels were observed with BD patients [43]. For this instance antidepressant treatment may significantly decrease plasma taurine levels. Amino acid values obtained for L-alanine, L-glutamine, L-ornithine, L-isoleucine, L-threonine,

Table 4. Amino acids quantitation in plasma from BD patients ($n = 9$) and comparison with similar studies [3, 4, 42–44]

Amino acid (μmol/L)	Current results (n = 9)		Mitani et al. [3] (n = 23)		Hoekstra et al. [4] (n = 5)		Hoekstra et al. [4] (n = 20)		Mayoral-Mariles et al. [42] (n = 17–21)		Altamura et al. [43] (n = 25)		Pinto et al. [44] (n = 5)	
	Average	SEM	Average	SEM	Average	SEM	Average	SEM	Average	SEM	Average	SEM	Average	SEM
L-Glutamine	574.3[a)]	50.3	393.4	40.4					583.4	28.4	447.0	65.2		
L-Alanine	314.7	23.8	404.4	42.4									273.0	11.0
Glycine	231.9	21.9	205.0	23.5	319.2	47.3	288.8	20.9	240.4	13.5	215.0	10.4	159.0	11.0
L-Threonine	127.3	8.2	155.2	17.2					80.9	5.4			84.0	2.0
L-Serine	101.1	6.0	107.4	9.6					93.1	3.6	108.0[b)]	4.2	153.0[b)]	9.0
L-Ornithine	84.3	7.4											97.0	2.0
L-Proline	54.5	5.7												
L-Isoleucine	53.7	4.4							44.9	2.5			71.0	11.0
Taurine	53.1	11.1	133.9	19.4							40.0	2.6	147.0	6.0
L-Glutamic acid	28.3[a)]	2.7	94.0	10.0	39.4	8.6	56.8	6.1	44.1	3.3	336.0	31.8	58.0	2.0
D-Serine	2.4	0.4	2.1	0.2										
Subject details	Inpatients with a diagnosis of bipolar I or II depression under lithium treatment		Patients with depression (17 out of 23 treated with imipramine drug)		Manic patients with bipolar I disorder under lithium		Manic patients with bipolar I disorder		Mild depressed elderly women		Bipolar and unipolar major depressed subjects		Patients with major depression diagnosed of anxiety disorder	

a) L-glutamine and L-glutamate values from 4 patients (collected between 2010–2011). Data presented are mean ± SEM.
b) Quantification of total serine.

and L-serine, reported here are in agreement with the literature [3, 4, 42–44]. Interestingly, most previously published articles reported total serine instead of the separate enantiomers.

4   Concluding remarks

We developed a sensitive CE-LIF method for the analysis of 14 amino acids in human plasma using 175 mM borate running buffer at pH 10.25, 12.5 mM β-CD, 10 s of sample injection (33 mbar) and L-2-aminoadipic acid as the internal standard. The method has been validated for 10 amino acids including some D- and L-enantiomers such as D- and L-serine, which cannot be detected with other general methods based on GC and HPLC. Validation parameters are adequate for bioanalysis. Undiluted human plasma samples can be processed by ultrafiltration, derivatized, and analyzed for amino acid profiles. The results obtained for representative amino acids plasma from BD patients are in agreement with literature values. The method is useful particularly in studies where plasma amino acid levels in patients are used as biomarkers for diagnosis of diseases, evaluating the disease progression, and monitoring response to drug therapy.

5   References

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